Metabolic Phases during the Development of Granulation Tissue

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1. The metabolism of incubated slices of sponge-induced granulation tissue, harvested 4–90 days after the implantation, was studied with special reference to the capacity of collagen synthesis and to the energy metabolism. Data are also given on the nucleic acid contents during the observation period. Three metabolic phases were evident. 2. The viability of the slices for the synthesis of collagen was studied in various conditions. Freezing and homogenization destroyed the capacity of the tissue to incorporate proline into collagen. 3. Consumption of oxygen reached the maximum at 30–40 days. There was evidence that the pentose phosphate cycle was important, especially during the phases of the proliferation and the involution. The formation of lactic acid was maximal at about 20 days. 4. The capacity to incorporate proline into collagen hydroxyproline *in vitro* was limited to a relatively short period at 10–30 days. 5. The synthesis of collagen was dependent on the supply of oxygen and glucose, which latter could be replaced in the incubation medium by other monosaccharides but not by the metabolites of glucose or tricarboxylic acid-cycle intermediates.

The life history of experimental granulation tissue can be divided into the following metabolic phases: (1) cell proliferation with a concomitant synthesis of nucleic acids and cell proteins; (2) specialized synthesis of collagen; (3) involution. This work was planned to characterize these metabolic phases *in vitro* and as a basis for further study of controlling mechanisms.

In the literature there is information on the nucleic acids of granulation tissue (Woessner & Boucek, 1961*a*; Viljanto & Kulonen, 1962; Williamson & Guschlbauer, 1963; Guschlbauer & Williamson, 1963; Bashey, Woessner & Boucek, 1964), on the enzymes (Woessner & Boucek, 1959, 1961*b*), on the collagen fractions (Jackson, 1957; Majaniemi & Kulonen, 1964; Viljanto, 1964) and on collagen synthesis (Green & Lowther, 1959; Boucek & Noble, 1961; Smirnov, Mazurov, Goncharova, Smirnov & Shkarenkova, 1964). However, systematic data are required on the metabolism of isolated slices at various intervals after the induction of the granulomas.

Preliminary reports of this work have appeared (Ahonen, Lampiaho & Kulonen, 1964; Kulonen, Lampiaho, Ahonen & Kari, 1964).

EXPERIMENTAL

Production of experimental granulomas. Male albino rats of the Wistar strain (130–200g.) were kept on a standard laboratory diet in wire-bottomed cages. The granulomas were induced by the implantation of four pieces of viscose-cellulose sponge (size 10mm.×10mm.×20mm. each, weight 75–85mg., sterilized in boiling water for 10min.) subcutaneously and symmetrically in the backs of the rats (Viljanto & Kulonen, 1962). After specified periods, the pieces with the ingrown granuloma tissue were dissected under ether anaesthesia and immersed in ice-cold 0·9% NaCl solution. The capsule around the sponge was removed and 0·5mm.-thick slices were cut with a Stadie–Riggs microtome in the cold room and weighed with a torsion balance.

Incubation. Most of the incubations were carried out at 37° in the flasks of a conventional Warburg constant-volume manometer. The standard shaking rate was 95 strokes/min. The centre well contained 0·2ml. of 5% (w/v) KOH (without filter paper). The flasks were incubated first for 15min. with the stopcocks open. Thereafter the oxygen consumption was read first after 15min. and later at longer intervals. The incubation was terminated after 3 or 6hr. by the addition of 0·2ml. of trichloroacetic acid solution (1g./ml.). For some incubations with larger amounts of tissue, Erlenmeyer flasks (25–50ml.) in a shaking incubator (A. Gallenkamp and Co. Ltd., London, E.C. 2) were used.

The incubation medium was based on Krebs–Ringer phosphate, made up as follows: 5·00ml. of 4·50% (w/v) NaCl solution, 0·20 ml. of 5·75% (w/v) KCl solution, 0·15ml. of 6·10% (w/v) $CaCl_2$ solution, 0·05ml. of 19·10% (w/v) $MgSO_4,7H_2O$ solution and 5·00ml. of 0·1M-phosphate buffer, pH7·4 (Umbreit, Burris & Stauffer, 1957). The following amino acids were added, each in 1ml. of aqueous solution, to the indicated final concentrations of L-form in the medium (Green & Lowther, 1959): DL-leucine, 0·76mM; L-isoleucine, 0·38mM; DL-valine, 0·85mM; DL-threonine, 0·84mM; DL-methionine, 0·13mM; arginine hydrochloride,

0·95mM; lysine hydrochloride, 1·10mM; L-tyrosine, 0·22mM; L-glutamine, 0·85mM. The volume of the final incubation medium was made up to 32·00ml. by the addition of 12·40ml. of water. Glucose was added dry to give a final concentration of 22·4mM.

Other compounds were added to the medium in 0·02–0·10ml. of water, or weighed as the solid. Their concentrations are given in Table 5. In some experiments glucose was replaced by other substrates (Tables 3 and 4).

In most of the experiments about 1 μc of [U-^{14}C]proline (specific activity 103 μc/mg.) was added in 0·1ml. of water. [6-^{14}C]Glucose (specific activity 23·0 μc/mg.) and [1-^{14}C]-glucose (specific activity 19·7 μc/mg.) were added in amounts of about 0·2 μc and 0·5 μc in 0·02ml. and 0·05ml. of water respectively. In these experiments the concentration of unlabelled glucose was 22·4mM, as usual. Sodium [1-^{14}C]pyruvate (specific activity 46 μc/mg.) was added as an aqueous solution (0·02ml. containing 0·2 μc). In the experiments on the utilization of glucose and pyruvate (Table 4) the medium contained both unlabelled 11·2mM-glucose and unlabelled 11·2mM-pyruvate and either 0·2 μc of [^{14}C]glucose or 0·2 μc of [1-^{14}C]pyruvate was added.

In most experiments, performed in duplicate or triplicate, five or six slices of granuloma tissue (each 60–120mg. fresh wt.) were placed in one Warburg flask, which contained altogether 3·1ml. of the incubation medium. In the experiments with labelled glucose a greater number of tissue slices was used (0·9–1·6g. fresh wt.).

Viability of the granuloma tissue. The effect of the conditions before the incubation were studied by incubating slices from 20-day granulomas. Two series of experiments were made to determine the significance of the time-interval before the incubation, the composition of the gas phase, the composition of the surrounding fluid and the ambient temperature (Table 1). The incubation period was 3hr. and it was terminated by the addition of an equal volume of 10% trichloroacetic acid solution. In this series the slices were washed first with five 5ml. lots of 5% trichloroacetic acid solution and then twice with diethyl ether–ethanol (2:1, v/v) and air-dried.

Attempts with homogenates. Granulomas were broken down with various revolving-blade homogenizers, with the Potter–Elvehjem apparatus, with ultrasonics, by grinding in a mortar with glass wool, by forcing them through small apertures in the frozen state, or by freezing and thawing. The remaining intact cells were removed by centrifugation at 600*g* for 15 min. The homogenates were diluted with Krebs–Ringer phosphate and incubated in the presence of [^{14}C]proline. No incorporation of ^{14}C was observed in any conditions.

Analytical methods. (*a*) Dry weight. The slices were washed with 0·9% NaCl solution (three 5ml. lots per piece) and once with water. The material was then dried at 105° overnight. The dry weight of the tissue was calculated by subtracting the weight of the dry sponge.

(*b*) Lactic acid. This was determined according to Barker & Summerson (1941).

(*c*) Nucleic acids. All the methods were based on the Schmidt–Thannhäuser procedure. A modification described by Fleck & Munro (1962) was employed. The estimations of RNA and DNA were based on the determination of ribose in the RNA fraction (Ceriotti, 1955) and on the determination of deoxyribose by the diphenylamine reaction (Burton, 1956).

A sponge with granulation tissue was homogenized in 5 vol. of water at 50000 rev./min. for 3min. in a homogenizer (E. Bühler, Tübingen, W. Germany). To remove the acid-soluble nucleotides 0·5 vol. of 2·1N-$HClO_4$ was added and, after a period of 15min., the suspension was centrifuged in a refrigerated centrifuge. The sediment was suspended in 0·7N-$HClO_4$ solution and collected again by centrifugation. This washing was repeated once. The hydrolysis of RNA was performed in 0·3N-KOH solution at 37° for 1½hr. The hydrolysate was neutralized with 10N-$HClO_4$ solution and excess of acid was added until its concentration was 0·5N. The precipitate was washed with 0·5N-$HClO_4$ solution by centrifugation and the supernatants were combined. They contained all the RNA but also some DNA. If the temperature was kept at 0°, the amount of contaminating DNA remained small (3–6% of total DNA). The precipitate, which contained the bulk of DNA, was solubilized by heating the suspended sediment in 5% (w/v) $HClO_4$ for 30min. at 90°. The remaining insoluble material was washed twice with 0·5N-$HClO_4$ and the supernatants were combined.

Because some DNA was present in the RNA fraction, both fractions had to be analysed by the diphenylamine reaction. The standard was prepared from DNA ('ex herring sperm') or from ribose. Attempts were made to isolate RNA from granulation tissue (see below), but it was considered more reliable to express the RNA as RNA ribose.

The duration of hydrolysis in 0·3N-KOH at 37° required to liberate nucleotides from RNA was checked in the range 1–23hr. A period of 1–1½hr. was found sufficient, as judged from the amount of liberated ribose, which reached the final value during the first hour. The recovery of ribose from isolated granuloma RNA was 13·3–14·7%. The E_{260} value increased continuously because of protein impurities, as described by Fleck & Munro (1962).

Prolongation of the hydrolysis with KOH caused a decrease in the total yield of DNA, especially in that fraction of DNA already solubilized by KOH hydrolysis and present as a contaminant in the RNA fraction.

We confirmed the results by Løvtrup (1962) that the yield of DNA was higher after hydrolysis at 70° than at 90°. After 15min. hydrolysis, when the yield was maximal, the difference was about 13%. It was also observed that the proportion of liberated DNA decreased with the weight of the sample. The washing of the insoluble residue must be thorough. Spongie fragments did not interfere with the analyses of nucleic acids.

Incorporated [^{14}C]*proline.* The determination of the hydroxy[^{14}C]proline in collagen was based on the procedure of Peterkofsky & Prockop (1962), but with the following changes: carrier imino acid was not added, the volume of toluene was reduced in some extractions to 20ml. instead of the original 30ml., and the concentration of the pyrophosphate buffer was 0·20M, because salts crystallized easily from the 0·25M-buffer originally suggested.

The collagen was isolated as gelatin and hydrolysed. The hydrolysate was oxidized with chloramine-T ('pro analysi') in two stages. First, the proline was converted into toluene-soluble Δ^1-pyrroline, while the corresponding derivative of hydroxyproline, pyrrolecarboxylic acid, remained water-soluble. On continued heating the pyrrolecarboxylic acid was decarboxylated and also became a toluene-soluble pyrrole.

The right amount of the oxidizing reagent, chloramine-T,

was checked. The complete extraction of pyrroline (from proline) with toluene was also checked and in our hands the extraction with ten 20 ml. lots of toluene brought the blank to a steady value, but after five extractions the blank was still four times as high. Toluene of 'pro analysi' grade was no better than the usual Erg. B 6 grade.

The values for proline in Fig. 4 refer to the first toluene extract. They have a limited significance, but are presented to allow temporal comparisons.

The standard was a sample of hydroxyproline, which was treated similarly through the oxidation and extraction steps. The recovery of peptide-bound hydroxyproline from pig-skin gelatin, containing 12·7% (w/w) of hydroxyproline, was about 74% (the samples were 1·2–4·9 μmoles) after hydrolysis.

The incubated tissue was treated by the procedure of Prockop, Udenfriend & Lindstedt (1961). The slices were homogenized in 20 ml. of water for 2–3 min. with the above-mentioned Bühler homogenizer with cooling with tap water. The insoluble material was collected by centrifugation (35000g for 15 min. in a refrigerated centrifuge). The sediment was rehomogenized in water with Potter–Elvehjem apparatus (size C; A. H. Thomas Co., Philadelphia, Pa., U.S.A.) and collected again by centrifugation. The sediment was dehydrated overnight in ethanol and then washed twice with diethyl ether–ethanol (2:1, v/v), air-dried with gentle heating and finally weighed. The washing of sediments with water and lipid solvents removed considerable amounts of nitrogenous substances.

The gelatinization was performed in water, first in an autoclave but later in sealed tubes, for 3 hr. at 120° (later at 130°). The gelatin solution was isolated by filtration. The insoluble matter was washed with seven 7 ml. volumes of warm water and the washings were added to the first filtrate. Gelatinization in trichloroacetic acid solution yielded less pure gelatin. The gelatin fraction contained about 15% of the total nitrogen. The hydroxyproline N in the gelatin fractions was 6·4% of the total N. One sample of the obtained gelatin was analysed for amino acid composition by the method of Spackman, Stein & Moore (1958); the amount of non-collagenous contaminants was judged to be small. About 6% of the total hydroxyproline remained in the sediment after gelatinization.

The combined filtrates were concentrated by evaporation on a boiling-water bath. Then HCl was added to a final concentration of 6 N. For the hydrolysis the acid solution was first kept in sealed tubes for 16 hr. at 124°, but later 3 hr. at 130° was found sufficient. The HCl was removed by evaporation on a boiling-water bath. The hydrolysates were decolorized with a small amount of charcoal.

Measurement of radioactivity. The fluid scintillation system NE 5503 (Nuclear Enterprises Ltd., Edinburgh) was used in all the experiments (voltage 675 v, input 50 mv). At least 1000 disintegrations or those during a 1 min. period were counted.

The radioactivities of the incubation fluids were measured by adding 0·05 ml. of the fluid to 2 ml. of NE 220 scintillation solution. The CO_2 was absorbed into primene (scintillation grade) of which 0·1 ml. was mixed with NE 213 scintillation solution (2 ml.) and 0·1 ml. of ethanol or methanol was added to clear the solution. The measured respiration was about 20% lower with primene than with KOH solution in the centre well.

The activity of proline and hydroxyproline was determined according to Prockop *et al.* (1961). The preparation of the samples is described in the previous section. To 10 ml. of toluene extract, which contained the imino acid derivatives, was added 1 ml. of scintillation fluid [15 g. of 2,5-diphenyloxazole and 50 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1000 ml. of redistilled toluene].

Reagents and chemicals. Glucose (monohydrate), galactose ('puriss'), fructose, glutamic acid, fumaric acid, succinic acid, citric acid, NaF, dinitrophenol (sodium salt), NaN_3, $FeCl_3,6H_2O$ ('pro analysi'), $CuCl_2,2H_2O$ ('pro analysi'), EDTA and methylene blue (B extra) were purchased from E. Merck A.-G. (Darmstadt, W. Germany), ribose was from L. Light and Co. Ltd. (Colnbrook, Bucks.), 2-oxoglutaric acid, trifluoroacetic acid ('purum'), GSH and aminoacetonitrile sulphate ('purum') were from Fluka A.-G. (Buchs SG, Switzerland), lactic acid (AnalaR) was from British Drug Houses Ltd. (Poole, Dorset), pyruvic acid (sodium salt) was from C. F. Boehringer and Soehne G.m.b.H. (Mannheim, W. Germany), β-hydroxybutyric acid (sodium salt) (lot 83B-0290) and 7-azaguanine were from Sigma Chemical Co. (St Louis, Mo., U.S.A.) and actinomycin D (LYO Meractino-mycin) was from Research Laboratories of Merck, Sharp and Dohme (West Point, Pa., U.S.A.). DNA ('ex herring sperm') and ribose were obtained from L. Light and Co. Ltd., and chloramine-T and L-ascorbic acid were from E. Merck A.-G.

DL-Leucine and L-isoleucine were obtained from L. Light and Co. Ltd., DL-valine and L-glutamine were from S. A. F. Hoffmann-La Roche Ltd. (Basle, Switzerland), DL-threonine and lysine hydrochloride were from Fluka A.-G. and DL-methionine, arginine hydrochloride and L-tyrosine were from E. Merck A.-G.

All the radioactive substances were supplied by The Radiochemical Centre (Amersham, Bucks.) and the scintillation fluids by Nuclear Enterprises Ltd. The scintillation chemicals, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene, were purchased from Fluka A.-G. The other chemicals were all of analytical grade or redistilled.

RESULTS

General development and viability of granulation tissue

Nucleic acids. The proliferation of the cellular material is reflected in the content of DNA and RNA (Fig. 1). The peak of both DNA and RNA is reached and cell proliferation is finished on the third or fourth week after the implantation. The time curve of the RNA content reflects the prolonged synthesis of non-collagenous proteins (Fig. 5).

Four attempts were made to prepare RNA from the granulomas, 21–30 days old, by the procedure of Kirby (1956, 1962), by using 15–45 granuloma pieces in each case. The content of nitrogen in the resulting preparations varied from 8·0 to 9·9% (w/w), that of phosphorus from 7·4 to 8·0% (w/w), that of protein as measured by the biuret reaction from 4·0 to 5·0% (w/w) and that of DNA as measured by the diphenylamine reaction from 13·4 to 28·8% (w/w). The E_{260}/E_{280} ratio was 1·75–1·88. The coefficients for the calculation of RNA from RNA ribose varied from 6·8 to 7·5, but if allowance

is made for the presence of DNA in these preparations it should be about 5.

The base composition of RNA with reference to the age of the granulation tissue has been investigated separately (Ahonen *et al.* 1964).

Viability. The viability was studied for practical reasons to learn how long the slices were usable (Table 1). The capacity for collagen synthesis by granuloma slices is best retained in the cold (not frozen) and in the presence of ascorbic acid but in the absence of oxygen, glucose and amino acids. It was found important to standardize the interval from the killing of the rats to the beginning of the incubation to 60 min. If the slices were kept in the ice-cold Krebs–Ringer phosphate solution for 1–4 hr., a decrease of about 7–12%/hr. was observed in the rate of oxygen consumption.

Viability of the tissue for collagen synthesis during homogenization was studied extensively as described in the Experimental section. The results were all disappointing. Indeed, other experiments in our Laboratory show that the polysome system of homogenized granulomas is easily degraded (Kumento, Kari, Hollmén & Kulonen, 1966) and this may be the reason why all our attempts to induce collagen synthesis with homogenized granuloma tissue were unsuccessful.

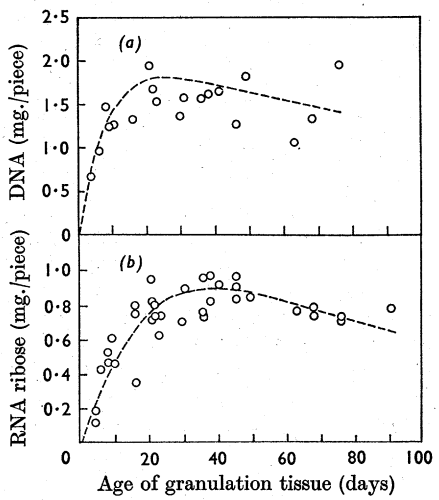

Fig. 1. Contents of (*a*) DNA and (*b*) RNA ribose in the granuloma pieces (2 cm.^{3}) during their development after implantation. The curves have been fitted visually.

Energy metabolism

Data on the consumption of oxygen are collected in Fig. 2. The utilization of glucose (data not presented) followed a similar course to the uptake of oxygen and the peaks fall on the same period as the maximal content of RNA, at four to five weeks. The oxygen consumption is not high in comparison with other tissues.

The formation of lactate (Fig. 3) differs from these patterns to some extent. There was a maximum at the same period as in the capacity for the synthesis

Table 1. *Viability of granulation tissue slices*

Each variable was studied separately in duplicate and the result expressed as a percentage with reference to the control sample that had been kept before the incubations in standard conditions (for 1 hr. in ice-cold incubation medium with air as gas phase). The incubations were performed as described in the Experimental section.

Conditions for the storage of slices before the beginning of the incubations	Incorporation of [^{14}C]proline (%)	
	Into collagen [^{14}C]proline	Into collagen hydroxy[^{14}C]proline
Duration:		
0 hr. (incubated immediately)	105·8	107·7
1 hr. (standard, if not stated otherwise)	100·0	100·0
2 hr.	98·2	84·6
Gas phase:		
Air (standard)	100·0	100·0
N_2	115·8	100·0
O_2+CO_2 (95:5)	108·2	79·4
Ambient solution:		
Incubation solution (standard)	100·0	100·0
Standard but without glucose and amino acids	152·2	106·1
Standard with added ascorbic acid (10 mM)	148·2	130·0
Medium of DiPietro & Weinhouse (1959)	130·3	75·4
Slices frozen at −18° for 24 hr.	45·8	0·8
Slices kept at 37° for 1 hr.	114·2	88·7

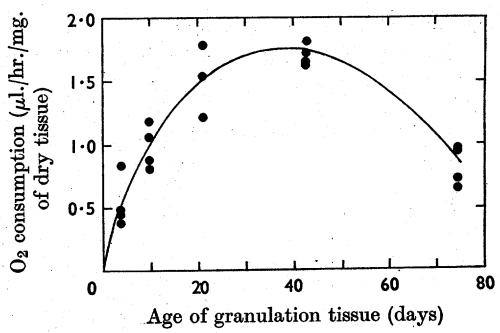

Fig. 2. Consumption of oxygen by slices of granulation tissue at the various phases of its development.

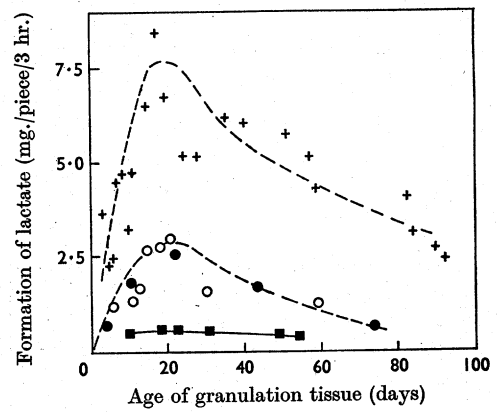

Fig. 3. Formation of lactic acid by slices of granulation tissue at the various phases of its development. +, About 0·5g. of slices in 3·0ml. of incubation medium; ○ and ●, two separate series with about 1·0–1·5g. of slices in 3·0ml. of incubation medium; ■, incubated without glucose.

of collagen (Fig. 4). As shown below, the availability of glucose was a condition for the synthesis of collagen (Table 3). For the manifestation of the maximal capacities both in the synthesis of lactate and also in the incorporation of labelled proline, the tissue weight/volume of medium ratio is rather significant.

For the estimation of the pentose phosphate cycle and glycolysis, the conversion of [1-^{14}C]-glucose and of [6-^{14}C]glucose into carbon dioxide was studied (Table 2). In addition to the data given in Table 2, the C-1/C-6 ratio can be calculated (from values obtained with Hyamine as absorbent) to be in the range 1·4–1·7 for 43-day granulomas. The ratio of carbon dioxide formation from [1-^{14}C]-glucose to that from [6-^{14}C]glucose reached the lowest values during active collagen synthesis. The ratio was much higher during cell proliferation (at 4 days) and slightly increased during the involution (at 74 days), indicating a higher proportion of carbon dioxide formed through the pentose phosphate cycle. The data in Table 3 confirm that glycolysis was a prominent feature in the metabolism of granulation tissue, especially in the synthesis of collagen. If glycolysis was inhibited by sodium fluoride or glucose replaced by pyruvate or lactate, [^{14}C]proline was not incorporated although pyruvate was utilized (Table 4). The role of different substrates is discussed in the next paragraph.

Capacity for collagen synthesis

Collagen synthesis during the life span of granulation tissue. In Fig. 4 data are collected from three different experimental series on the capacity of the tissue to incorporate [^{14}C]proline into collagen hydroxyproline. The form of the curve differs markedly from that of the curves plotting oxygen consumption and the content of nucleic acids. The period when the tissue is capable of collagen synthesis *in vitro* is relatively short. The accumulation of collagen in granulation tissue is completed by the end of the first month (Viljanto, 1964).

Fig. 5 shows the incorporation of [^{14}C]proline into collagen [^{14}C]proline and also into non-collagenous protein. The absence of hydroxy[^{14}C]-proline from the non-collagenous fraction is evidence for the complete extraction of collagen. Non-collagenous protein is synthesized from proline during the whole observation period and also after the synthesis of collagen had ceased.

The net dry weight has been used as the basis for the calculations. The determinations made by us agreed with the data of Viljanto (1964): the maximum of the net dry weight is reached at 21 days and it is thereafter followed by a slow decline. If the data in Fig. 4 were calculated on the basis of the amount of sponge (indicating the volume of the tissue) the peak would be even more prominent.

Effect of experimental modifications. Several experiments, all performed with slices from 20–22-day granulomas, were standardized by taking the incorporation of [^{14}C]proline into collagen hydroxy[^{14}C]proline in the respective control experiments as 100%. The significance of various energy-yielding substrates is presented in Table 3. Glucose could be replaced by galactose, and to some extent by fructose or β-hydroxybutyrate, but not by pyruvate, lactate or tricarboxylic acid-cycle metabolites. In many experiments the incorporation of [^{14}C]proline into collagen [^{14}C]proline was measured also and it was much less affected by the absence of glucose. The results of the experiments where glucose and pyruvate were used together show that the effect of pyruvate cannot be explained by a dilution of proline precursors by pyruvate.

Table 2. *Utilization of* [^{14}C]*glucose by incubated granulation tissue slices*

The amount of added [1-^{14}C]glucose varied from 159000 to 726000 counts/min., that of [6-^{14}C]glucose from 142000 to 652000 counts/min. The wet weight of the granuloma pieces, including the sponge, varied from 876 to 1695 mg.

Age of the granulation tissue (days)	Expt. no.	Position labelled	Production of labelled CO_2: Counts/min./mg. of dry tissue	Production of labelled CO_2: C-1/C-6 ratio
4	1	C-1	14·1	20·1
		C-6	0·7	
	2	C-1	21·5	10·7
		C-6	2·0	
10	1	C-1	5·9	0·7
		C-6	8·2	
	2	C-1	10.0	1·0
		C-6	10·5	
21	1	C-1	11·5	1·8
		C-6	6·4	
	2	C-1	6·7	1·8
		C-6	3·7	
74	1	C-1	23·7	2·8
		C-6	8·6	
	2	C-1	10·0	3·0
		C-6	3·3	

Table 3. *Effect of the substitution of glucose by other substrates in the incubation fluid on the collagen synthesis by incubated granulation tissue slices*

The number of experiments is given in parentheses. The age of the granulomas was 20 days. The results are expressed as percentages with reference to the incorporation in the control sample with glucose as substrate, included in every set of experiments.

Substrate	Incorporation of [^{14}C]proline into collagen hydroxy[^{14}C]-proline (%)
Glucose (22·4 mM) (28)	100·0
Without glucose (2)	44·1
Galactose (22·4 mM) (2)	105·2
Fructose (22·4 mM) (2)	79·0
Ribose (22·4 mM) (2)	55·6
α-Oxoglutarate (44·8 mM) (2)	10·8
Glutamate (44·8 mM) (2)	11·4
Fumarate (44·8 mM) (2)	6·9
Succinate (44·8 mM) (2)	5·2
Citrate (44·8 mM) (2)	6·6
Lactate (44·8 mM) (2)	7·5
Pyruvate (44·8 mM) (2)	13·5
Pyruvate (10 mM) + glucose (22·4 mM) (2)	102·3
Pyruvate (10 mM) + glucose (22·4 mM) + NaF (1·0 mM) (2)	58·0
NaF (1·0 mM) + glucose (22·4 mM) (2)	7·8
Fluoroacetate (1 mM) + glucose (22·4 mM) (7)	95·4
β-Hydroxybutyrate (44·8 mM) (2)	67·5

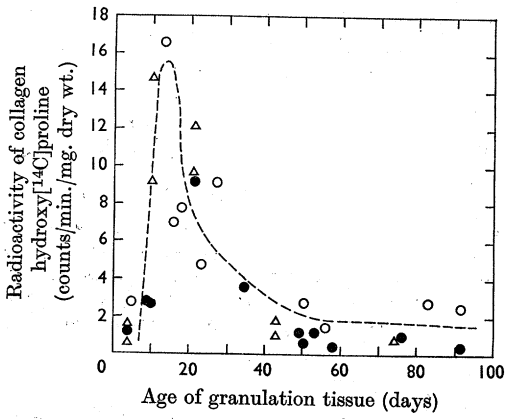

Fig. 4. Capacity for biosynthesis of collagen from [^{14}C]-proline by slices of granulation tissue at the various phases of its development. The different symbols indicate data from three different series of experiments.

The data in Table 4 show that pyruvate was taken into the cells and metabolized to carbon dioxide. The ratio of pyruvate and glucose as sources of carbon dioxide was 5:1, which agrees well with the data of Cremer (1962) for brain and kidney. When crystallized insulin was added to the medium, the incorporation of [^{14}C]proline into collagen increased by 40–50%, but the corresponding incorporation in other proteins less (Mikkonen, Lampiaho & Kulonen, 1965).

Exploratory experiments were made to determine the effect of various additions or modifications in the medium (Table 5). Glucose was always present. The data confirm the importance of an abundant supply of oxygen. In one experiment with nitrogen

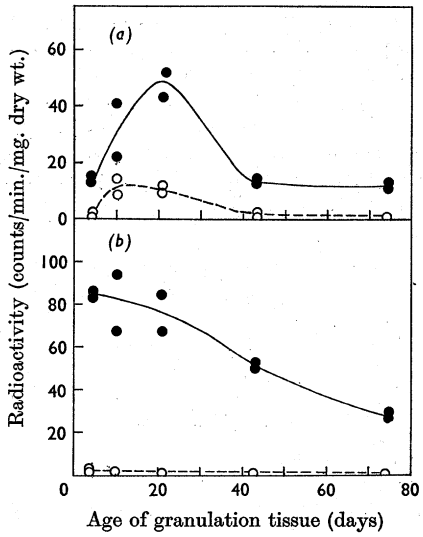

Fig. 5. Incorporation of [^{14}C]proline into various fractions of slices of granulation tissue at the various phases of the development: (*a*) gelatinized collagen; (*b*) non-collagenous protein. ●, Incorporated [^{14}C]proline; ○, incorporated hydroxy[^{14}C]proline. The ordinate gives the radioactivity in the first toluene extract.

Table 4. *Comparison of glucose and pyruvic acid as the sources of carbon dioxide in the incubation of granulation tissue slices*

The age of the granulation tissue was 20 days. The added radioactivity varied from 283500 to 391000 counts/min. and the dry weight of the granulation tissue from 108 to 117mg.

Substrate	Formation of $^{14}CO_2$/mg. (% of added radioactivity)
[6-^{14}C]Glucose	0·0027
[6-^{14}C]Glucose	0·0040
[6-^{14}C]Glucose	0·0035
[1-^{14}C]Pyruvate	0·011
[1-^{14}C]Pyruvate	0·025
[1-^{14}C]Pyruvate	0·018

as the gas phase the incorporation of [^{14}C]proline into collagen hydroxy[^{14}C]proline decreased to 1·3%, but its incorporation into collagen [^{14}C]-proline was decreased only to 21·3% and into non-collagen [^{14}C]proline to 4·5%. In an oxygen–carbon dioxide mixture the incorporation into collagen hydroxy[^{14}C]proline increased to 140·8% and that into collagen [^{14}C]proline to 122·4%, but that into non-collagen [^{14}C]proline decreased to 85·4%.

Azaguanine and actinomycin D had modest effects only, as also did methylene blue and Fe^{3+} and Cu^{2+} ions. It is remarkable that aminoacetonitrile does not affect the collagen synthesis in the present conditions at all. Homogenates from various embryonic tissues or granulomas had a marked repressing effect on the incorporation of [^{14}C]proline into collagen in the slices.

Table 5. *Effect of the modifications and additions to the medium on collagen synthesis by incubated granulation tissue slices*

Glucose was present in all the experiments. The age of the granulation tissue was 20 days. The number of the experiments is given in the parentheses. The results are expressed as percentages with reference to the incorporation in the control sample, incubated without modifications and included in every set of experiments.

Addition or modification	Incorporation of [^{14}C]proline into collagen hydroxy[^{14}C]-proline (%)
Standard conditions (28)	100·0
Pure O_2 as gas phase (2)	132·3
O_2+CO_2 (95:5) as gas phase (4)	148·1
N_2 as gas phase (4)	8·8
2,4-Dinitrophenol added (0·1mM) (1)	52·6
2,4-Dinitrophenol added (1·0mM) (1)	5·3
NaN_3 added (0·1mM) (1)	67·4
NaN_3 added (1·0mM) (1)	34·8
GSH added (1·0mM) (2)	98·1
Fe^{3+} added (0·1mM) (2)	88·9
Cu^{2+} added (1·0mM) (2)	80·6
EDTA added (1·0mM) (2)	70·0
Methylene blue added (20 μM) (2)	95·4
Methylene blue added (10 μM) (4)	67·0
Azaguanine added (10mM) (2)	91·7
Actinomycin D added (0·5mg./ml.) (2)	70·5
Aminoacetonitrile added (1·0mM) (5)	102·2

DISCUSSION

Metabolic phases. It is remarkable that in adult mammalian tissue a phase of the synthesis of a specific protein can be sharply defined. The first, proliferative, phase is characterized by a high rate of the synthesis of nucleic acids and non-collagenous proteins and by a relative importance of the pentose phosphate cycle. The content of DNA in rat cells is about 5·7 $\mu\mu$g./cell (Brawerman & Shapiro, 1962). From the maximal content of DNA/2cm.^{3} piece (about 1·7mg.) it is inferred that the number of cells is, at the saturation phase, about 150×10^6/cm.^{3} and thus one cell 'occupies' a volume of about 6000 μ^3.

During the second phase the synthesis of RNA and non-collagenous protein continues but at a decelerated rate. The main characteristic is the ability to synthesize collagen. From the work of

Viljanto (1964) we may calculate that the accumulation of collagen ends when about 7 mg./cm.^{3} has been formed. One fibroblast can be estimated to form about 40–50 $\mu\mu$g. of collagen. Nothing is known about the factors that limit the period of collagen synthesis which does not correlate temporally with the concentration of RNA, utilization of oxygen or synthesis of other proteins but correlates to some extent with the formation of lactate.

During the third phase, when cells no longer synthesize collagen, there is still an appreciable number of cells present. There may be an invasion of cells other than fibroblasts, when the pentose phosphate cycle regains importance.

Williamson & Guschlbauer (1961, 1963) found that the rate of RNA formation reached its peak at 7–9 days in the regenerating wound tissue. Similarly, the highest rates of synthesis of RNA and collagen were observed at 8–10 days and at 28–29 days after implantation (Smirnov *et al.* 1964). Assuming that the number of cells is proportional to the amount of DNA, there was a continuing decrease of cellular RNA from 8 to 20 days in subcutaneously implanted polyvinyl sponge. Continued synthesis of extractable RNA, at a time when no further increase of DNA was occurring, implied a turnover of a part of RNA (Bashey *et al.* 1964). Woessner & Boucek (1961*a*) found that the DNA content rose to a maximum value in implanted polyvinyl sponge at 20 days and then remained constant. Collagen accumulation reached the final value at 36 days. Jackson (1957) states that in carrageenin granuloma the maximal content of collagen was observed at 21 days. These findings, as also those of Viljanto & Kulonen (1962) and Viljanto (1964), are in agreement with the present results.

Regulation of the collagen synthesis. There are two identified factors that limit the synthesis of collagen during the active period, namely the supply or utilization of oxygen and the presence of glucose. The importance of glucose is corroborated by the enhancing effect of insulin. Woessner & Boucek (1959) observed that the enzymes involved in anaerobic glycolysis, in the tricarboxylic acid cycle and in the hexose monophosphate shunt, were present with activities of similar magnitude at 24–27 days.

The oxygen in the hydroxyl group of hydroxyproline originates from atmospheric oxygen (Prockop, Kaplan & Udenfriend, 1963; Fujimoto & Tamiya, 1963), and aerobic conditions are necessary for collagen synthesis in slices (Kao, Hitt, Dawson & McGavack, 1963). α-Oxoglutarate is also reported to be necessary for proline hydroxylase (Hutton, Tappel & Udenfriend, 1966), but in our conditions it did not support the incorporation of proline into collagen. A requirement of oxygen is evident also in the cell-free system (Peterkofsky & Udenfriend, 1963). It is not known how much the hydroxylation of the peptide intermediate limits the synthesis of collagen in granuloma. The significance of the oxygen supply has been elaborated further *in vivo* (Niinikoski, Penttinen, & Kulonen 1966). Increasing the oxygen concentration in the gas breathed by rats resulted in improved tensile strength both in healing skin wounds and in granulomas.

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